Vol. LXVI, No. 1 February, 1934

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE TROPHIC NATURE OF CHLOROGONIUM AND CHILOMONAS [1]

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Introduction

The application of pure culture methods has done much to further our knowledge of protozoan nutrition. Growth of organisms in synthetic media has been accomplished largely through a process of trial and error, and very often success has been attributable to the wide range of adaptability which many of these forms exhibit. Some of the green flagellates may be either completely autotrophic in light, utilizing inorganic nitrogen and carbon dioxide, or totally heterotrophic in darkness. While some of the plant-like flagellates in particular are capable of existence under such extremely varied conditions, the great majority of protozoa are restricted to a more limited food environment. The purpose of the following experiments is to determine the trophic nature of *Chlorogonium euchlorum* Ehrenberg, *C. elongatum* Dangeard and *Chilomonas paramecium* Ehrenberg when grown in bacteria-free cultures, *i.e.*, to determine their capability of autotrophic existence in light and their heterotrophic nutrition in darkness. *Chilomonas*, being colorless, normally assimilates soluble organic food, although claims for its completely autotrophic nature have recently been advanced by Mast and Pace (1932). Jacobsen (1910), working with *Chlorogonium euchlorum* in bacterized cultures, believed this form to be autotrophic in light as well as heterotrophic in darkness, although he was unable to control cultural conditions adequately to determine this fact with certainty. Grateful acknowledgment is due Professor R. P. Hall for suggestions during the investigation.

[1] Part of a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy at New York University, June, 1933.

Material and Methods

Pure lines of *C. cuchlorum* and *C. elongatum* were obtained from the German University at Prague through the courtesy of Professor E. G. Pringsheim, while the bacteria-free strain of *Chilomonas paramecium* was derived from a single organism isolated from pond water at Woods Hole during the summer of 1932. Cultures of the three species were maintained in culture tubes in a 0.25 per cent tryptone-salt medium at pH 7.0.

Before it can be concluded that a certain nutritional factor in a given medium is or is not utilized by the organism concerned, it is necessary, according to Lwoff (1932), to subculture that species at least three or more times in order to eliminate growth effects produced by compounds present in the original stock medium transferred with the inoculum and in order to allow for utilization of reserve food materials. Accordingly, conclusions stated below are based only on cultures which have been carried through a number of transfers, the cultures having been maintained in constant light at a temperature of 28° C. in a thermostat-controlled water bath. In each series of transfers, 6–12 culture tubes containing the same volume of medium were inoculated equally from a dilution flask (Loefer, 1934). After incubation transfers to similar media were made; this procedure was continued until growth ceased or until the possibility of continued growth was established. The following media at pH 7.0 were used in the respective experiments designated later.

Medium A:

NH_4NO_3	0.5 gram
KH_2PO_4	0.5 "
$MgSO_4$	0.1 "
NaCl	0.1 "
$FeCl_3$	trace
Distilled water	1 liter

Medium B:

KNO_3	0.5 gram
KH_2PO_4	0.5 "
$MgSO_4$	0.1 "
NaCl	0.1 "
$FeCl_3$	trace
Sodium acetate	2.5 gram
Dextrose	2.0 "
Proteose-peptone	2.5 "
Distilled water	1 liter

Medium C:

KH_2PO_4	0.5 "
$MgSO_4$	0.1 "
NaCl	0.1 "
$FeCl_3$	trace
Sodium acetate	2.5 gram
Distilled water	1 liter

Bacteriological tests for purity of the cultures were carried out at regular intervals during the course of each experiment. The sodium acetate used was obtained from the Eastman Kodak Co., Rochester, N. Y., while tryptone, peptone, and dextrose were products of the Difco Laboratories.

Autotrophic Nutrition

Tubes containing medium A were inoculated with *C. euchlorum* from a dilution flask and placed at a north window at room temperature. At the end of four weeks the cultures were intensely green. Consecutive transfers were made at four-week intervals for forty weeks, at the end of which time it was evident that autotrophic existence in light is possible indefinitely in the medium used. Homologous series on *C. elongatum* showed the same result.

The object of another group of experiments was to determine whether growth is more rapid in a solution containing nitrates or in one containing ammonium compounds. For this purpose medium C was used as a base. Equal lots containing 0.5 per cent concentrations of NH_4NO_3, KNO_3, NH_4Cl and $(NH_4)_2SO_4$, respectively, were tubed and sterilized in the regular manner. After inoculation (1.0 cc.) with *C. euchlorum,* cultures were grown at room temperature near a north window. After fifteen days no significant differences in growth were evident, as determined by the Sedgwick-Rafter counting-cell method. Four successive transfers were made in each type of medium; subsequent growth indicated that this species uses either nitrate or ammonium to equal advantage. The results for *C. elongatum* were similar. No appreciable pH changes occurred during the culture periods.

Chilomonas paramecium has been reported capable of using ammonium nitrogen (Mast and Pace, 1932). In an attempt to verify this observation the same types of media as were used above for *Chlorogonium* were inoculated with *Chilomonas.* A number of the cultures were kept at room temperature and some at a constant temperature of 28° C. in moderate light. Growth occurred in all of them after four or five days. A second transfer was made, but only slight growth was noted in a few of the tubes after an equal period of time. No growth took place at all in cultures of the third transplant, even after a longer period of time. Even when these media contained 0.05–0.10 per cent Na_2SiO_3 in addition to the regular constituents, *Chilomonas* did not survive a third transfer. *Chlorogonium,* likewise, will not grow in darkness on inorganic nitrogen, even in the presence of sodium acetate and silicate.

Heterotrophic Nutrition

Since it was impossible to grow *Chlorogonium* in darkness in a medium containing inorganic nitrogen only, a medium containing several different carbon and nitrogen compounds was used (medium B). Sterile tubes of this solution were inoculated with *C. euchlorum* on November 10, 1932, and incubated in total darkness at room temperature. Subinoculations were made at intervals of approximately four weeks, and to date the organisms have been transferred 11 times and the cultures are still green, indicating that growth and continued synthesis of chlorophyll is taking place. Parallel experiments on *C. elongatum* indicate the same result. Although the organisms appear somewhat smaller than when grown in the usual stock medium in light, their normal color and continued division indicate that indefinite growth without photosynthesis is possible when the culture medium contains adequate nitrogen and carbon sources.

Discussion

In the group of chlorophyll-bearing flagellates, most species utilize either nitrate or ammonium nitrogen in light, using CO_2 as a carbon source. Among the Phytomonadida which are facultatively autotrophic, various nitrogen sources produce a differential effect in amount of growth. Lwoff (1932) found that *Hæmatococcus pluvialis* grew better with ammonium salts than with nitrates, although *Chlamydomonas agloëformis* grew equally well in either medium. The group Euglenida likewise exhibits specific growth differences (Dusi, 1933), some forms (*Euglena gracilis, E. stellata, E. klebsii*) growing equally well with ammonium salts or nitrates as nitrogen sources. *E. anabæna* is more limited since it is unable to utilize nitrate, but grows well on ammonium salts. Others (*E. pisciformis, E. deses*), although generally considered totally photosynthetic organisms because of their green color, are actually mixotrophic since they cannot utilize inorganic nitrogen. These few instances are indicative of the variations found among chlorophyll-bearing flagellates. The results obtained for *Chlorogonium* (*euchlorum, elongatum*) mark them as truly autotrophic species since they can utilize both ammonium and nitrate nitrogen. The wide range of adaptability of this genus, shown by its ability to grow heterotrophically as well as autotrophically, no doubt accounts for Jacobsen's (1910) conclusion that it was primarily mixotrophic rather than autotrophic in its food habit.

Only in one case has truly autotrophic nutrition been claimed for a colorless flagellate. Mast and Pace (1932) state that, " Chilomonas can consequently, without light, synthesize carbohydrates, fats, proteins, and

protoplasm from inorganic compounds, obtaining nitrogen from NH_4 and carbon from CO_2." Such synthesis is effected in an inorganic salt medium containing Na_2SiO_3 either with or without sodium acetate. In the present investigation with bacteria-free cultures of *Chilomonas paramecium* there was no indication that inorganic nitrogen could be utilized, with or without Na_2SiO_3 in the medium, even when sodium acetate was supplied as an organic carbon source. Growth which occurred in the first and second subcultures was probably due to the small amount of organic nitrogen transferred with the inoculum from the stock medium. Inasmuch as these results have been reported only in abstract form, it is at present impossible to repeat their experiments on this strain, using their exact method.

It would appear (Cleveland and Collier, 1930) that certain strains of *Leptomonas* can be cultured in dextrose and other carbohydrate solutions which contain no nitrogen source. From the description of the methods used, it seems highly probable that the small amount of nitrogen necessary for growth was transferred from the stock medium with the inoculum. It is extremely doubtful that these forms would have survived successive transfers in this medium, since growth without nitrogen is unknown in any other protozoa.

Under favorable conditions of organic nutrition many green, otherwise autotrophic, organisms have been cultivated in total darkness for varying periods of time. Jacobsen's (1910) results on *Chlorogonium* were mentioned above. A race of *Euglena gracilis* has been grown saprophytically for several years in total darkness by Lwoff (1932), having, however, lost its green color in the meantime. *Chlorogonium* (*euchlorum, elongatum*) differs in that it still retains its green color after a year of cultivation in total darkness, although the chloroplasts appear somewhat modified.

Summary

Chlorogonium (*euchlorum, elongatum*) is capable of indefinite autotrophic growth in a mineral medium in light, growth in nitrate being as good as in cultures containing ammonium salts. It was impossible to maintain bacteria-free cultures of *Chilomonas paramecium* indefinitely by subculturing them at regular intervals in a medium containing inorganic nitrogen, even in the presence of sodium acetate as a carbon source. Since November 10, 1932, both species of *Chlorogonium* have been cultured in total darkness in a medium containing mineral salts, sodium acetate, dextrose and proteose-peptone without losing their green color.

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